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Characteristic distribution pattern of *Helicobacter pylori* in dental plaque and saliva detected with nested PCR

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The precise mode of transmission and the natural reservoir for *Helicobacter pylori* are unknown. PCR assays have proved to be highly sensitive and specific and are regarded as the method of choice for detecting *H. pylori* DNA in the oral cavity. The aim of this study was to investigate the prevalence and distribution of *H. pylori* in the oral cavity. Forty-two patients undergoing gastroscopy were investigated for the presence of *H. pylori* in dental plaque and saliva by nested PCR, and in the stomach by the ^{13}C-urea breath test. Samples tested comprised dental plaque from molars, premolars and incisors and saliva. Two sets of primers homologous to the 860-bp fragment of *H. pylori* DNA, which have been shown previously to be highly sensitive and specific, were used for nested PCR. Eleven patients (26.2%) were infected with *H. pylori* in the stomach. *H. pylori* DNA was identified in dental plaque samples from 41 patients (97%) and in 23 saliva samples (55%). The prevalence in dental plaque from molars, premolars and incisors was 82%, 64% and 59%, with an odds ratio of 3.18, 1.24 and 1 (reference), respectively. In conclusion, *H. pylori* was present in the oral cavity of 97% of tested patients, with a characteristic distribution that was independent of the infection status of the stomach. Thus *H. pylori* may belong to the normal oral microflora.

Introduction

Helicobacter pylori infection is one of the most common bacterial infections in man [1]. The infection is widely accepted as an important cause of gastritis and is strongly associated with peptic ulcer disease and gastric cancer [1, 2]. Despite numerous studies, the hypothesis that the oral flora may be a permanent reservoir of viable *H. pylori* is still controversial [3–18]. Furthermore, studies have failed to show an increased risk of *H. pylori* infection in dentists [16]. However, this finding may support the fact that *H. pylori* infection is predominantly acquired in early childhood.

Studies that employed culture methods may have underestimated the prevalence of *H. pylori* in the oral cavity. One reason may be the presence of viable but non-culturable coccoid *H. pylori* organisms [19]. In this regard, PCR assays have a much greater sensitivity. However, results are controversial and detection of *H. pylori* has ranged from 0% to 100% because of the use of different PCR primers with samples from different patient groups [6–16]. As these results are not in agreement with the prevalence of *H. pylori* infection in the stomach, it is questionable as to whether they represent the real prevalence of this micro-organism in the human oral cavity or are artifacts of the methods applied. A recent study demonstrated that different primers had different detection limits and specificity for *H. pylori* added to dental plaque or saliva, and primers directed to the 860-bp fragments of *H. pylori* DNA showed the greatest sensitivity and specificity compared with primers directed to the 16S rRNA gene or the urease A gene [20].

It has been shown that different anatomic sites of the oral cavity favour the growth of different bacterial species [21]. A preferred intra-oral habitat has not been identified for *H. pylori*. Therefore, this study investigated the prevalence of *H. pylori* in dental plaque samples from different locations (molar, premolar and incisor) and in saliva.

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Patients and methods

Subjects and sampling

Forty-two patients (15 female, 27 male; mean age 40 years) who underwent upper gastrointestinal endoscopy because of dyspeptic complaints were enrolled in this study. These patients had no history of antibiotic treatment with proton pump inhibitors, H_2-blockers, antacids or bismuth compounds in the 3 months before sampling, to avoid interference with *H. pylori* detection methods. Before endoscopy, supragingival dental plaque samples were scraped from molars, premolars and incisors and transferred separately into tubes containing physiological saline. Sterile curettes were used for each collection site to prevent cross-contamination between dental plaque samples from different locations in each patient. In the case of patients without molars, premolars or incisors, dental plaque samples were pooled for each subject. Saliva was collected from each patient after they had chewed a paraffin wax capsule for 1 min. Dental plaque samples and saliva were frozen immediately and stored at −20°C until required for DNA extraction.

Informed consent was obtained from each patient and the study was approved by the Ethics Board of the University of Ulm.

^{13}C-urea breath test

Active infection with *H. pylori* was determined by breath testing with carbon-13 as described previously [22]. An initial breath sample was collected in a plastic bag. The patients then received 200 ml of apple juice containing 75 mg non-radioactive ^{13}C-urea (Mass Trace, Woburn, MA, USA). A second breath sample was collected 30 min later. The breath samples were analysed with an isotope selective non-dispersive infrared spectrometer (Wagner Analytical Systems, Bremen, Germany). An increase in the ratio of $^{13}CO_2$ to $^{12}CO_2$ between the baseline sample and the 30-min sample of $> 4/1000$ was considered to indicate active infection.

Preparation of genomic DNA for PCR

DNA was extracted from dental plaque or saliva as described previously [6]. Briefly, 1 ml of dental plaque suspension or saliva was centrifuged in a conventional table-top centrifuge for 10 min at 7000 rpm. Pellets were suspended in 600 μl of digestion buffer (0.1 M NaCl, 0.01 M Tris-HCl, pH 8.3, 0.25 M EDTA, sodium lauroylsarcosine 1%) containing proteinase K 100 μg/ml and incubated at 55°C for 3 h. DNA was extracted with an equal volume of phenol-chloroform and precipitated with isopropanol. The DNA pellet of each sample was washed with ethanol 70% and resuspended in 50 μl of sterile water. Extensive care was taken to avoid contamination during all steps of collecting and preparing the samples.

PCR primers

The primer set EHC-U (5′-CCCTCACGCCATCAGTCCCAAAAA-3′) and EHC-L (5′-AAGAAGTCAAAAACGCCCCAAAAC-3′), homologous to the 860-bp fragment of *H. pylori* genomic DNA (80 076–80 492 bp), produces an expected 417-bp (80 076–80 492 bp in *H. pylori* genomic DNA) PCR product [6]. Based on the sequence of the 860-bp fragment of *H. pylori* genomic DNA, an additional pair of primers ET-5U (5′-GGCAAATCATAAGTCCGCAGAA-3′) and ET-5L (5′-TGAGACTTTCCTAGAAGCGGTGTT-3′), internal to the fragment amplified by EHC-U/EHC-L, was used and the expected product size was 230 bp (80 198–80 427 bp in *H. pylori* genomic DNA) [20]. The oligonucleotides were synthesised at Interactiva Biotechnology GmbH (Ulm, Germany) and purified by reversed-phase HPLC.

PCR amplification

The first round of amplification consisted of 40 cycles comprising a 45-s denaturation step at 94°, a 45-s primer annealing step at 59°C and a 30-s primer extension step at 72°C. For the initial cycle, before the addition of Taq polymerase, the denaturation step was 5 min at 95°C. For the final cycle, primer extension was extended to 10 min. All reactions were performed in a final volume of 25 μl, which contained 10 PCR buffer 2.5 μl, deoxynucleoside triphosphate mixture 0.5 μl (final concentration, 0.2 mM each dATP, dCTP, dGTP and dTTP; Pharmacia Biotech, USA), 25 pM of each primer, template DNA 10 μl and Taq polymerase (GibcoBRL, Life Technologies) 1.25 U. The 10 PCR buffer, optimised with the Stratagene Opti-prime PCR optimisation kit, contained 100 mM Tris HCl (pH 9.2), 15 mM $MgCl_2$ and 750 mM KCl. The profile of the second round of amplification was similar to the first round, except that 0.2 μl of the first round product served as template for the second PCR and only 25 amplification cycles were used. Positive and negative controls were included for each batch of amplification. The DNA of *H. pylori* (type strain ATCC 43629) served as a positive control. Water and a ‘negative sample control’ were used as negative controls. The former contained all the necessary components except template DNA and the latter contained physiological saline without sample and had been included in all the DNA preparation steps. Extensive care was taken to prevent false positive results due to contamination, i.e., the use of fresh disposable devices; preparation of template DNA, pre- and post-PCR materials in separate locations; gloves were changed frequently, and other instructions outlined by Kwok and Higuchi were followed [23]. Amplification was performed with a thermocyler (TRIO-Thermoblock, Biometra, Gottingen, Germany). The PCR products were analysed by agarose gel electrophoresis with ethidium bromide staining for visualisation.

Specificity of primers EHC-U/EHC-L and ET-5U/ET-5L

Southern hybridisation was used to identify products amplified from dental plaque or saliva samples of 20 randomly selected patients by single-step PCR with primers EHC-U/EHC-L. Ten μl of the PCR reaction mixture were analysed by electrophoresis on agarose gels. Then amplified DNA fragments were transferred to a nylon membrane (Amersham, Life Science) and hybridised to a specific probe as described previously [24]. The specific probes were produced by PCR from *H. pylori* type strain ATCC 43629, purified from gels with the QIAquick gel extraction kit (QIAgen) and labelled with (^{32}P)-dCTP with a random primer labelling kit (Rediprime, Amersham, Life Science).

Statistical analysis

The χ^2 test and odds ratio were used to analyse differences in the prevalence of *H. pylori* in dental plaque and saliva and were calculated with the SAS software package [25].

Results

Specificity of primer EHC-U/EHC-L and ET-5U/ET-5L

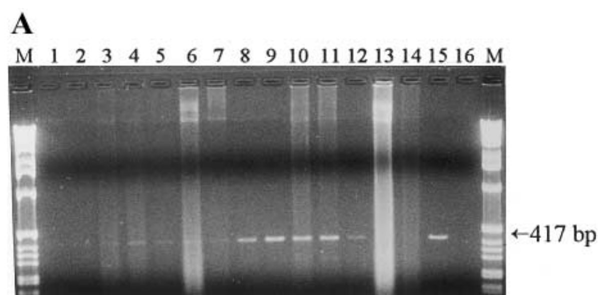
Southern hybridisation revealed that all 417-bp bands from tested dental plaque were positive and gave results consistent with those from nested PCR. Some representative results are shown in Fig. 1.

H. pylori in dental plaque samples

Overall, 11 (26%) of 42 patients had an active *H. pylori* infection as measured by the ^{13}C-urea breath test; 41 (97%) of the 42 patients were *H. pylori*-positive in at least one dental plaque sample. The only patient with a negative PCR result had teeth missing and only a little plaque material could be collected. Overall, from the 117 dental plaque samples collected from all patients, 80 (68%) were positive for *H. pylori* DNA. Three patients were excluded from further analysis because they had missing teeth. Of the 39 patients from whom plaque samples could be collected from all three locations, *H. pylori* DNA was detected in one location in 11 (28%) patients, in two locations in 15 (39%) patients and in all three locations in 13 (33%) patients. Fig. 2 shows representative PCR results for the dental plaque and saliva samples.

H. pylori DNA was detected in all three locations, but with great differences in prevalence: 82% (32 of 39) in the molar region, 64% (25 of 39) in the premolars and 59% (23 of 39) in the incisors. Table 1 shows that the odds ratio of having a positive PCR result for *H. pylori* DNA in the molar region was 3.18 (95% CI, 1.01–10.26) compared with the incisors (reference).

Fig. 2. Nested PCR amplification of DNA from dental plaque and saliva for detection of *H. pylori*. Lane **M**, 1-kb DNA ladder; **1–5**, dental plaque; **6–8**, saliva; **9**, positive control (ATCC 43629); **10**, negative control (containing all the necessary components except template DNA); **11**, negative control (containing physiological saline without sample), which had been processed through all DNA preparation steps before PCR amplification and was then PCR-amplified.



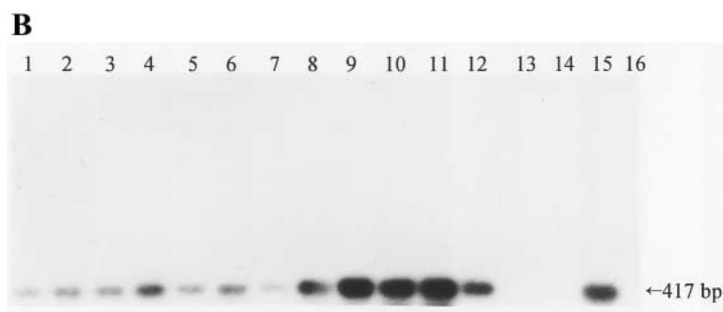


Fig. 1. Detection of *H. pylori* DNA in dental plaque specimens by a single-step PCR with primers EHC-U/EHC-L. The amplified products were analysed by agarose gel electrophoresis (**a**) and Southern hybridisation (**b**). Lanes **1–12**, positive samples; **13** and **14**, negative samples; **15**, *H. pylori* type strain ATCC 43629 as a positive control; **16**, negative control; **M**, 1-kb ladder.

Table 1. Localisation of *H. pylori* DNA in supragingival plaque from sites in the oral cavity of 39 subjects by nested PCR

Site	Positivity (%)	Odds ratio (95% CI)
Incisor	59.0 (23/39)	1 (reference)
Premolar	64.1 (25/39)	1.24 (0.45–3.43)
Molar	82.1 (32/39)	3.18 (1.01–10.26)

H. pylori in saliva samples

Overall, *H. pylori* DNA was detected in 55% (23 of 42) of the saliva samples. *H. pylori* DNA was detected in the saliva of 64% (7 of 14) of patients with active *H. pylori* infection in the stomach, compared with 52% (16 of 31) of patients without *H. pylori* infection ($p > 0.2$).

Discussion

The results of the present study show that *H. pylori* is present in most dental plaque samples, independent of the infection status of the stomach. This is the first report indicating such a high prevalence in developed countries. Similar results have been reported from developing countries [3–5]; however, most of the previously published results from developed countries that used PCR showed lower detection rates [10–17]. A study by Banatvala *et al.* in patients attending an endoscopy clinic in London demonstrated 63% correlation between *H. pylori* in the stomach and in dental plaque samples [15]. Primers with different sensitivity and specificity and samples from different patient groups may be responsible for these discrepancies. The primers EHC-U/EHC-L and their target 860-bp DNA used in this study have been shown to be highly sensitive and specific for *H. pylori* [6, 26]. A recent study showed that nested PCR increased sensitivity and specificity, and that primers directed to the 860-bp DNA of *H. pylori* were more sensitive and specific for *H. pylori* added to dental plaque or saliva than primers directed to the urease A gene or 16S rRNA gene [20]. A single-step PCR with the primers EHC-U/EHC-L and 40 cycles of amplification consistently detected five *H. pylori* cells added to saliva or to dental plaque [20]. In contrast, others reported the detection limit to be 10–100 cells of cultured *H. pylori* [6, 8, 27, 28]. The possibility of false positivity is very unlikely in the present study, because considerable care was taken to prevent contamination, as described by Kwok and Higuchi [23]. The high detection rate in the present study may argue for the assumption that *H. pylori* belongs to the normal flora of the oral cavity in man. However, as the PCR method used detects only a part of the *H. pylori* DNA, the absolute number of viable *H. pylori* organisms may be low.

In all, 55% of the patients in the present study were positive for *H. pylori* DNA in saliva, a rate similar to that reported by Li *et al.* who found *H. pylori* DNA in 59% of the patients with the same primers [6]. Furthermore, Li *et al.* found that subjects with gastric *H. pylori* infection had a higher prevalence of *H. pylori* in saliva than those without the infection [6]. However, the difference was not statistically significant in the present study. Recently, Yiang *et al.* reported similar results [29]. They found that all saliva samples from five patients who were negative for *H. pylori* in the stomach were positive for *H. pylori* DNA by a highly specific nested PCR.

Nguyen *et al.* found that the distribution of *H. pylori* in the oral cavity was not uniform. They took two samples of dental plaque from two different sites in each patient, but detected *H. pylori* in only one sample [12].

The results of the present study confirm a specific distribution pattern for *H. pylori* in the oral cavity, with a higher prevalence in plaque from molars than from premolars or incisors. This distribution may be in accordance with the micro-aerophilic characteristics of *H. pylori*. Theoretically, oxygen exposure may decrease gradually from incisors to molars, favouring the growth of *H. pylori* in the molar region. Plaque composition varies from site to site and in response to various oral influences. Similarly, the growth of *H. pylori* in the oral cavity may be influenced by various factors, such as temperature, pH, oxidation-reduction potential, the availability of nutrients, salivary flow and antimicrobial substances [30, 31]. Each factor may influence the selection of oral micro-organisms in a given location and support the growth of a bacterial community with a characteristic composition [32]. Therefore, it seems to be important to collect dental plaque samples from different sites in the oral cavity.

It is not known why only a small proportion of the patients harbouring *H. pylori* in the oral cavity have an active infection in the stomach. This study investigated only adult patients. As *H. pylori* infection is mainly acquired in childhood [33], and the number of *H. pylori* in the mouths of adults is very low, this does not seem to be a significant risk for hosts themselves [34, 35]. According to a recent study, it seems likely that an infected mother may play a key role in transmission of *H. pylori* to her child, possibly *via* saliva [36]. A similar oral–oral transmission from mother to child has been shown for *Streptococcus mutans* and for *Actinobacillus actinomycetemcomitans* [37, 38]. However, the factors that are responsible for the typical growth behaviour and colonisation pattern of *H. pylori* in the oral cavity have still to be defined.

In conclusion, *H. pylori* was present with characteristic distribution in oral cavity of almost all the subjects studied, suggesting that *H. pylori* may belong to the normal microflora of the adult oral cavity. There seems to be no correlation between the prevalence of *H. pylori* in dental plaque samples from different locations in the oral cavity and saliva and infection in the

stomach in adult patients. Further studies should investigate this association in children.

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